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ON SEXUAL AGGLUTINATION AND MATING-TYPE SUBSTANCES (GAMONES) IN ISOGAMOUS HETEROTHALLIC CHLAMYDOMONADS. II. THE EFFECT OF CONCANAVALIN A UPON THE MATING-TYPE REACTION[1]

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In the copulation of isogametes in *Chlamydomonas*, the initial contact between sexually different cells at fertilization occurs spatially localized in the mating-type reaction, a species-specific agglutination of the flagella tips. The responsible surface components, the mating-type substances, have been purified and act, in their isolated state, as isoagglutinins: the male component causes homosexual agglutination between gynogametes and the female component isoagglutinates androgametes (*cf.* Wiese, 1965, 1969). In these isoagglutinations, the initial contact between sexually different gametes is isolated in copy form and can be studied in the interaction between the isoagglutinins and the reacting gametes. Pairs of mating-type substances have been isolated from two sexually compatible and four sexually isolated taxons (*C. eugametos* and *C. moewusii* syngen I; *C. moewusii* syngen II, *C. reinhardti, C. mexicana,* and *C. chlamydogama*). Both isoagglutinins of a species are sex- and species-specific glycoproteins of complex nature. The physiological and chemical analysis of the isoagglutinins concentrates on their functional structure, particularly on the nature of their combining sites, on their mode of interaction, and on those features which condition the specificity of the agglutination mechanism as to sex and species.

The phytohemagglutinin Concanavalin A, a globulin of the jack bean *Canavalia ensiformis,* combines specifically with non-reducing terminal sugar residues of branched macromolecules (*cf.* Sumner and Howell, 1936; Goldstein, Hollerman and Merrick, 1965; Goldstein and So, 1965; So and Goldstein, 1968). Concanavalin A (CONA) seems to react exclusively with α-D-glucopyranosyl, β-D-fructofuranosyl, and α-D-mannopyranosyl residues. Unmodified hydroxyl groups on C_3, C_4 and C_6 are considered to be essential for establishing the contact with the combining site of the CONA-molecule (Goldstein, Hollerman and Smith, 1965; Goldstein and Iyer, 1966; So and Goldstein, 1967). The interaction is known to depend on bivalent cations (Agrawal and Goldstein, 1968). Analogous to an antibody, the binding capacity of CONA is inherent to two combining sites (So and Goldstein, 1968). Hence, CONA is able to form complexes with and to cause precipitation of polysaccharides, mucopolysaccharides, lipopolysaccharides and glycoproteins possessing those sugars in a terminal position. This substrate specificity of its binding action makes CONA an analytical tool for the char-

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acterization of functional components of carbohydrate nature. In addition, however, CONA may combine in an unspecified manner with various polyelectrolytes and with certain neutral polysaccharides which do not possess the terminal sugars mentioned (*cf.* Doyle, Woodside and Fishel, 1968). CONA was checked for its potency to react with the glycoproteinaceous components of the sexual agglutination mechanism in *Chlamydomonas*, the mating-type substances, and for its ability to provide information on the character of their combining sites.

Material and Methods

The effects of CONA on the mating-type reaction have been investigated in a test system with two sexually compatible taxons [*C. eugametos* (Indiana Collection Nos. 9 and 10, *cf.* Starr, 1964) and *C. moewusii* syngen I (Indiana Coll. Nos. 96 and 97)] and a third form, *C. moewusii* syngen II (Indiana Coll. Nos. 792 and 793), which is sexually isolated from the first two pairs of mating types. On the homologies between the mating types see below.

The cultivation of the organisms, the sexualization of the vegetative cultures, *i.e.*, the induction of gametogenesis, the preparation and purification of the isoagglutinins, and the test techniques have been described earlier (*cf.* Wiese, 1965).

The CONA preparation used was the commercially available product from Calbiochem (Los Angeles). CONA was applied in solutions of 0.02 *M* TRIS-buffer pH 7.2 or in 0.002 *M* phosphate buffer pH 6.7. Trypsin (salt-free, twice crystallized) (Worthington Enzymes, Freehold, New Jersey) was applied in 0.05 *M* TRIS-buffer at 26° C for 45 min at pH 7.76. The trypsin-inhibitor (Soy bean inhibitor, 5 × crystallized) was obtained from Nutritional Biochemical Corporation (Cleveland, Ohio). Experimental details are given in the text.

Results

When gametes of *C. eugametos*, *C. moewusii* syngen I and *C. moewusii* syngen II are brought into a solution of 0.01% CONA, an instantaneous isoagglutination within each of the six gamete types occurs. In their typical form, the clusters of agglutinated gametes cannot be distinguished from normal sexual clumping between andro- and gynogametes, from the two isoagglutinations induced by the isolated mating-type substances (isoagglutinins), and from those isoagglutinations (Baker and Wiese, 1968) which are caused by antibodies against isolated mating-type substances. In addition to true isoagglutinations from flagella tip to flagella tip, there may occur a cluster formation in which the participating gametes adhere by means of their flagella tips to microscopically visible particles which have adsorbed CONA as, for instance, detached flagella. Such a reaction can be induced by coating sephadex beads with CONA molecules to which the gametes of all six sexes adhere. Uncoated sephadex does not adsorb any of the six gamete types. The ability of cells to combine with CONA can hence easily be judged in a system with CONA-coated sephadex beads.

The causation of clustering by isoagglutination and the unilateral inhibition of one mating-type activity, described below, are independent of another CONA-action on the cell's motility causing immobilization after 5–45 minutes, depending on the concentration of CONA and varying with the physiological state of the cells. The immobilization also ensues in both sexes of all three taxons.

When gametes of the three taxons are incubated with lower concentrations of CONA (0.001%), they no longer enter isoagglutination except in a few but typical clusters. If these incubated gametes (after being washed twice by centrifugation and resuspension in CONA-free medium) are combined with untreated gametes of their opposite sex, a clear-cut difference between the two sexes appears. Treated gynogametes of *C. eugametos* agglutinate with untreated androgametes to an undiminished degree, whereas absolutely no agglutination occurs in the reciprocal combination between treated androgametes and untreated gynogametes.

Such unilateral inhibition of one mating-type activity also exists within the two pairs of mating types of *C. moewusii* which are preliminarily designated as

The Sensitivity of the Mating Type Activities to 0.001% Concanavalin A and 0.1% Trypsin.

species / sex	C. eugametos		C. moewusii syngen I		C. moewusii syngen II	
	♂	♀	+	−	+	−
Concanavalin A Sensitivity	S	R	S	R	S	R
Trypsin Sensitivity	R	S	R	S	R	S

FIGURE 1. The sensitivity of the mating-type activities to 0.001% concanavalin A (30 min incubation) and 0.1% trypsin (45 min incubation); S = sensitive; agglutinability entirely blocked or destroyed. R = resistant; no detectable diminution of the agglutinability. For further explanation see text.

syngens I and II. The final designation of the two forms, which are sexually isolated by a nonmatching system of mating-type substances, will be made after their incompatibility mechanism has been analyzed in greater detail. The form which we call syngen I (Indiana Collection Nos. 96/97; Starr, 1964) is sexually compatible with *C. eugametos* (Indiana Collection Nos. 9/10); the latter's male sex (No. 9) being homologous to the (+) sex in *C. moewusii* syngen I (No. 96) (*cf.* Wiese, 1965). It is the (+) sex in *C. moewusii* syngen I which is sensitive to CONA. The sexual bipolarity for syngen II of *C. moewusii* (Indiana Collection Nos. 792/793, *cf.* Wiese and Metz, 1969) was fixed by declaring strain No. 792 as (+) sex because its cells continue the flagella beat and move the vis-à-vis pair after papilla fusion of the two mating partners [as does the (+) sex in *C. moewusii* syngen I (Lewin, 1952, 1954) and the male sex in *C. eugametos* (Wiese and Jones, 1963)]. Also in this taxon one sex only, and it is again the (+) sex, is sensitive to CONA.

This unilateral sensitivity of the agglutination mechanism to CONA in the three taxons is reciprocal to their unilateral sensitivity to 0.1% trypsin. Trypsin destroys the capacity to agglutinate in the female sex of *C. eugametos* and in the corresponding (−) sex of *C. moewusii* syngen I whereas the complementary sexes

are trypsin-resistant (Wiese and Metz, 1969). Checking the two sexes of *C. moewusii* syngen II with 0.1% trypsin revealed also in this form, as in the other two taxons, only one of the two sexes sensitive, and it is again the (−) strain, *i.e.* that sex which is CONA-insensitive. As in *C. eugametos* and *C. moewusii* syngen I, the sexual inactivation of the gynogametes by trypsin is prevented by 0.05% trypsin inhibitor. Gynogametes of all three forms, sexually inactivated by trypsin, still possess their capacity to be adsorbed to CONA-coated sephadex.

All these effects of CONA in all three forms can be reversed by addition of 0.5% mannose. The various data for all three taxons are given in Figure 1.

The Agglutination Pattern of the Various Gamete Types with the Different CONA–isoagglutinin Complexes

Test Gamete Type		CONA–isoagglutinin Complexes*					
		C. eugametos		C. moewusii syngen I		C. moewusii syngen II	
		♂	♀	+	−	+	−
C. eugametos	♂	O	A	O	A	O	O
	♀	O	O	O	O	O	O
C. moewusii syngen I	+	O	A	O	A	O	O
	−	O	O	O	O	O	O
C. moewusii syngen II	+	O	O	O	O	O	A
	−	O	O	O	O	O	O

FIGURE 2. The agglutination pattern of the various gamete types with the different concanavalin A—isoagglutinin complexes. The concanavalin-complexes of the taxons and sexes above were checked for their capacity to cause agglutination with the gamete types at the left; A = agglutination, O = no agglutination.

The normal sexual agglutination between andro- and gynogametes, and the two isoagglutinations caused by the isolated mating-type substances are not reversible by mannose.

From the supernatants of androgametes and of gynogametes, the sex-specific isoagglutinins are precipitated quantitatively by 0.1% CONA. The male and female precipitates, washed twice by centrifugation and resuspension in distilled water in order to remove unadsorbed CONA, act differently when combined with test gametes: in the complex formed with CONA, the male or (+) component has entirely lost its capacity to agglutinate gynogametes, whereas the precipitated female or (−) substance continues to extend its combining sites to androgametes. Precipitated at an excess of isoagglutinin, there is no detectable agglutination of either complex due to uncovered combining sites of CONA with the gametes of the homologous sex, or with both gamete types of the respectively incompatible species (Fig. 2).

Discussion

The capacity to combine specifically with terminal sugars meeting certain specifications makes CONA a discriminating tracer for functions connected with polysaccharides or with the carbohydrate moiety of complex molecules. The mating-type reaction in isogamous *Chlamydomonas* species involves an instantaneous agglutinative adhesion between sexually different gametes. According to the analyses of the isoagglutinins which represent isolated mating-type substances (*cf.* Wiese, 1965, 1969), the contact function is inherent to components of glycoprotein nature (Förster, Wiese and Braunitzer, 1956; Wiese, 1961, 1965, 1967, 1969). Whatever the type of surface to surface interaction may be, the responsible contact mechanism is distinguished by the promptness and specificity of its reaction. A special requirement exists for the presence of bivalent cations (Ca^{++}, Mg^{++}) (Lewin, 1954; Tsubo, 1961; Wiese and Jones, 1963). Details of the mating-type reaction reveal that the agglutination mechanism in dioecious *Chlamydomonas* species must operate on the basis of a complementarity between sex-specific components, must be located on or associated with the flagella tip, and must be biosynthesized or set into action during gametogenesis (*cf.* Wiese, 1969). In addition, the mechanism must be responsible for the absolute species specificity of gamete contact. In accordance with the general concept on sex cell contact as an antigen-antibody like relation (*cf.* Metz, 1967), we assume that the bipolar sexual difference between the two gamete types is based upon a complementarity between components standing in mutual receptor relationship. In *Chlamydomonas*, we encounter the problem that the two isoagglutinins do not neutralize each other *in vitro* (Wiese and Wiese, 1965).

The sexual complementarity between andro- and gynogametes can be unilaterally addressed by CONA: combination of CONA with the terminal sugars on the flagella surface of androgametes (*i.e.*, with the mating-type substances *in situ*) and on the isolated male mating-type substance (the male isoagglutinin) totally removes the agglutinability. A corresponding complex formation with CONA also occurs with the female components as documented by the CONA-induced isoagglutination of gynogametes, by the specific attachment of gynogametes to CONA-coated sephadex, and by the precipitation of the female isoagglutinin. In the female sex, however, the combination of CONA with the terminal sugars does not interfere with the component's capacity to agglutinate. The female receptor sites involved in sexual agglutination, are not identical with the CONA-adsorbing sites, and, in addition, both areas do not overlap in a manner that the sexual receptor site is sterically blocked by the CONA-adsorption to the sugars. In accordance with this, tryptically inactivated gynogametes still adsorb CONA. This interpretation of the CONA-effect requires additional confirmation of the special role ascribed to the sugars, since the inactivation of one sex might be caused by an unspecified complex formation of CONA with the contact component due to its polyelectrolyte nature.

In both sexes of all three taxons, the complex formed with CONA can be dissociated by mannose restoring in all three types of androgametes the unaltered capacity to agglutinate. Of the individual sugars which are the same in all six isoagglutinins (rhamnose, xylose, arabinose, galactose and mannose), only mannose splits the CONA-complex with the mating-type substances. Mannose was

identified by Dr. P. Weber, Department of Biochemistry, State University of New York at Buffalo. The proper mating-type reaction between andro- and gynogametes, and the two isoagglutinations caused by a form's isoagglutinins are not prevented or reversed by mannose.

The reciprocal, unilateral interference of trypsin with the mating-type reaction does not necessarily indicate the direct inactivation of a proteinaceous receptor. Further characterization by blocking protein-specific functional groups and more data on the chemical composition and on the functional architecture of the female isoagglutinin are needed to confirm the protein nature of the female receptor site.

In addition to the sex-discriminating effect of CONA, its reaction with *Chlamydomonas* gametes demonstrates how an interaction between appropriate macromolecules in the cell's surface can account for a cell contact as instantaneous as is typical for the mating-type reaction. A compound such as CONA even shares those structural properties of the mating-type substances which provide them, in their isolated state, with the capacity to cause isoagglutination. The data suggest that, at normal sex cell contact, a proteinaceous component on one gamete type with combining sites similar to those of CONA might interact with a complementary sugar-containing component on the other gamete's surface.

A common dependency of the interaction of CONA with sugar-containing macromolecules (Agrawal and Goldstein, 1968) and of the mating-type reaction on bivalent cations further supports the parallelism between both events. On the basis of a similar relation, Rybak and Burstein (1960) concluded that, since fertilizin agglutinates with lipoproteins in the sera of mammals in the presence of Ca^{++}, a lipid component on the sperm might interact with the fertilizin of the egg in the Ca-dependent gamete contact at sea urchin fertilization. For *Chlamydomonas* (*cf.* Wiese, 1961), the further analysis of the male mating-type substance and of the effect of glycosidases upon androgametes will decide whether the two Ca^{++} actions need exclude one another. In addition, Ca^{++} in the observed concentrations may well be engaged in functions other than direct contact making (*cf.* Köhler, 1956).

In addition to its promptness, a most impressive feature of sex cell contact is the absolute specificity governing this event in a manner that no trial and error attempts are initiated between gametes of the same sex or between incompatible gamete types. Either the contact ensues instantaneously—within part of a second, a certain cell density provided—or not at all. Affecting the bipolar agglutination mechanism in each form unilaterally, neither CONA nor trypsin observes the incompatibility barrier between the three taxons, revealing that some basic sexual surface bipolarity exists in each form which is alternatively eliminated by the two agents. The features which condition the species specificity of the agglutination mechanism must be overimposed on this bipolarity, modifying it in a manner that the bipolarity comes to bear only within the matching pattern of the right system.

Summary

Concanavalin A (CONA) isoagglutinates the gametes within each sex of *Chlamydomonas eugamctos*, *C. moewusii* syngen I, and *C. moewusii* syngen II by a typical agglutinative adhesion of the flagella tips. Incubation of gametes with

lower CONA-concentrations which do no longer cause isoagglutination, effects blockage of the mating-type activity in only one of the two sexes, the other one being unaffected. In the three taxons investigated, it is always the androgametes which are sensitive to CONA. In addition, CONA immobilizes the cells after a short period of time. The CONA-induced isoagglutinations of both gamete types, the immobilization, and the unilateral blocking of one mating-type activity can be reversed entirely by mannose. In the three taxons, the unilateral sensitivity of the mating-type reaction to CONA is reciprocal to a unilateral sensitivity to 0.1% trypsin. Gynogametes, sexually inactivated by trypsin, still interact with CONA. In each of the three taxons, CONA precipitates both sexual isoagglutinins but only one of them, that of the CONA-insensitive sex, in active form. The data are discussed as to their bearing on the nature of the initial contact mechanism between sex cells.

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